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Use of a Bioluminescent *Pseudomonas aeruginosa* Strain within an In Vitro Microbiological System, as a Model of Wound Infection, To Assess the Antimicrobial Efficacy of Wound Dressings by Monitoring Light Production▽

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A bioluminescent *Pseudomonas aeruginosa* was incorporated into an in vitro static diffusion method to determine whether light output could be used as a measure of wound dressing efficacy. A significant linear correlation was observed between viable counts and bioluminescence during exponential growth in planktonic culture ($r^2 = 0.969$). Exponential-phase cells were used to inoculate cellulose discs for integration into an in vitro wound model that incorporated a reservoir of serum. A significant linear correlation was found between bioluminescence (photon counts monitored by a low-light camera) and viable counts in this growth environment ($r^2 = 0.982$). Three antimicrobial wound dressings were applied to the surface of freshly prepared sample discs within the wound model, and the kill kinetics were codetermined by photon and viable counts. Quantifiable kill rates gave the same order of efficacy for the three wound dressings using both types of measurement, and a significant linear correlation was shown between photon and viable counts ($r^2 = 0.873$) within this killing environment. Under all defined conditions, a significant linear correlation between bioluminescence and viable counts was shown but the actual slope of the correlation was different, depending on the physicochemical environment of the cells. Hence, significantly more light per cell ($P < 0.0001$) was produced when cells in discs were exposed to a killing environment compared to a growing environment. As long as defined conditions are employed, the resulting linear correlation enables the state of the system to be continually monitored without disturbance, allowing more immediate and accurate calculations of kill rates without the need for viable counting.

Within the field of wound care, it is essential to be able to study the antimicrobial effectiveness of conventional or novel types of wound dressings. It has long been known that wound infection can delay wound healing and cause increased morbidity and associated health care costs (3), and this has led to the introduction of medicated dressings. Due to concerns over the increased incidence of microbial resistance particularly associated with nosocomial infections, antimicrobial-impregnated dressings tend to utilize broad-spectrum antiseptic agents and numerous methods have been described to assess their antimicrobial efficacy (13, 24, 30).

We have previously described a simple in vitro static diffusion method using cellulose discs (31) that is both reproducible and allows for accurate determination of kill rates of wound dressings tested against aerobes, anaerobes, and yeasts, giving it definite advantages over existing in vitro microbiological systems as models of wound infection. This method has been further developed, incorporating a reservoir of serum within a hydrogel polymer test bed, bathing the test microbes in protective serum proteins and providing a more realistic pattern of controlled fluid donation akin to a moderately exuding wound (10). However, the main disadvantages of these culture-dependent methods are that they are very labor intensive, due to the taking of discontinuous time-sample readings that require viable counting (with the associated high cost in consumables), and each reading is disruptive, so that no single sample disc can be continually monitored throughout the course of the experiment. In addition, preliminary experiments are often required to determine which sample time points are the most appropriate in order to capture the kill rates.

The use of bioluminescent organisms as reporters of various microbiological phenomena is well documented (11). Light emission in bacteria is coordinated by the *lux* genes; *luxA* and *luxB* code for subunits of a luciferase enzyme which produces light from the oxidation of a reduced flavin mononucleotide ($FMNH_2$) and a long-chain aldehyde (RCHO), produced by a biochemical pathway involving *luxCDE* gene products (23). If the full complement of *lux* genes is inserted into a receptive bacterial strain, under the control of a constitutive promoter, then light will be continually produced. The production of light within genetically modified strains is dependent on gene transcription and oxygen concentrations, as well as the reducing power available within the cell. In a study of abrupt decline of luciferase activity (ADLA), when a cell encounters nutrient starvation (19), it was concluded that NAD(P)H regeneration (reducing power) was essential for light production and that this was likely to be the main rate-limiting step. This hypothesis has been supported by other experiments which have shown that, by indirectly inducing NADPH accumulation in *Escherichia coli*, luminescence was enhanced (9). This demonstrates the dependence of the biochemical pathways that result in bioluminescence on the production of ATP and NADPH and

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hence their intimate association with the Krebs cycle, electron transport, and fatty acid/riboflavin synthesis (9): thus, bioluminescence can be considered to be a monitor of cell metabolism.

Bacteria engineered to express the *lux* genes have been used to monitor pathogenesis and growth both in vitro (4) and in vivo (5), using the constitutive production of light as a reporter of viable, metabolically active cells. Furthermore, the quantifiable use of luminescence as a measure of cell viability is reported to show a good correlation with standard recovery techniques such as viable counting under defined conditions (2, 17). In the development of one animal wound model, it was shown that there was a good correlation between photons and viable counts both in vitro and in vivo, and in general, the relationship appeared to be independent of the presence or absence of the test antibiotics (27). Numerous other studies have also shown the suitability of using whole-cell bioluminescent reporter strains for in situ determination of the precise inactivation kinetics (pharmacodynamics) of antibacterial agents (12, 16, 18, 20). However, in vitro studies have shown that, although in general the relationship between bioluminescence and viable counts is closely correlated, there are sometimes discrepancies. Moreover, these can be analyzed so as to discriminate the effects of a given antimicrobial agent in terms of either inhibiting multiplication or metabolism (1, 23, 28).

The main objective of this study was to incorporate a bioluminescent *Pseudomonas aeruginosa* reporter strain into a modified version of an existing in vitro wound model (10) to determine the relationship between photons and viable counts. If a significant correlation could be found, then a method of testing wound dressings by monitoring bioluminescence alone could be developed, removing the need for viable counting. The ability to continually monitor the state of the system, without taking physical samples that potentially disturb it, gives the potential for determination of more immediate and accurate calculation of the dynamic kill kinetics relating to the antimicrobial efficacy of wound dressings.

MATERIALS AND METHODS

Bacterial strains and growth reagents. *P. aeruginosa* PAO1 SEI (ATCC 15692) was previously transformed with a recombinant plasmid containing the *luxCDABE* gene cassette of *Photorhabdus luminescens* (22), producing the strain *P. aeruginosa* MCS5-lite. *P. aeruginosa* PAO1 SEI was maintained on nutrient agar, and *P. aeruginosa* MCS5-lite was maintained on nutrient agar containing gentamicin (10 mg/liter) (Sigma-Aldrich, Dorset, United Kingdom), the selective agent for the recombinant plasmid.

Planktonic growth of *P. aeruginosa* MCS5-lite. Overnight cultures of *P. aeruginosa* MCS5-lite were grown in 1% tryptone–0.5% yeast extract (TYE) broth containing gentamicin (10 mg/liter) and used to inoculate 50 ml of broth of the same composition within a 250-ml flask to a standardized optical density at 540 nm (OD_{540}) of 0.02. Flasks were then incubated at 32°C with orbital shaking at 200 rpm (model S150; Stuart Shakers, United Kingdom), and four replicate cultures were used to follow the growth kinetics of the luminescent strain. At various time intervals over 24 h, samples were removed and used to determine OD_{540} (Helios; Thermo Electron Corporation, United Kingdom), viable counts, and bioluminescence using a single-tube luminometer (Junior LB9509; Bertech, Germany).

Growth of *P. aeruginosa* MCS5-lite within an in vitro wound model. An exponential-phase cell culture of *P. aeruginosa* MCS5-lite was grown in a planktonic culture and serially diluted 1 in 1,000. Cells were harvested from 10 ml of cell culture by centrifugation at 8,000 × *g* for 5 min, washed, and resuspended in a mixture of 1 ml TYE (in the absence of gentamicin) plus 1 ml fetal calf serum (FCS) (Biosera, Ringmer, United Kingdom). Cellulose discs (1 cm^2) were then inoculated with the test cell suspension, whereby each disc contained approximately the same number of viable cells (10^4 CFU/cm^2). Microbe-laden test discs were placed and distributed evenly onto the surface of preprepared hydrogel polymer test bed assay plates (HTPs) incubated with FCS as previously described (10). Growth was monitored over the course of 24 h by following both photon and viable counts for four replicate experiments. Photon count readings per disc were recorded every hour for the first 12 h and at 24 h by placing samples beneath an intensified charge-coupled device (ICCD) photon-counting camera (model 225; Photek, United Kingdom) mounted onto a blacked-out incubator set at 32°C using a 60-s integration time at each time point. The numbers of photons per sample were expressed as photons/cm^2 since the discs have a surface area of 1 cm^2. For viable counts, hourly samples for the first 12 h and a 24-h sample were taken by removing a disc to a 10-ml volume of sterile phosphate-buffered saline (PBS) and vortex mixing for 1 min to release cells into suspension. Neat suspensions were serially diluted 1:10 to $1:10^{-2}$ in PBS. Viable counts were then performed of neat and diluted suspensions using a spiral plater (Don Whitley Scientific, Shipley, United Kingdom) onto nutrient agar recovery medium containing gentamicin (10 mg/liter) and incubated at 37°C aerobically for 24 h to determine the numbers of CFU per sample (CFU/cm^2).

Integration of *P. aeruginosa* MCS5-lite into an in vitro wound model to determine antimicrobial wound dressing efficacy. Two iodine-releasing wound dressings, Iodozyme (dressing 1; Insense Ltd., Bedford, United Kingdom) and Inadine (dressing 2; Johnson and Johnson, Skipton, United Kingdom), and one silver-releasing wound dressing, Acticoat Absorbent (dressing 3; Smith & Nephew Medical Ltd., Hull, United Kingdom), were tested against *P. aeruginosa* MCS5-lite within a modified version of the in vitro static diffusion method (Fig. 1) (10). Exponential-phase cell cultures of *P. aeruginosa* MCS5-lite cell cultures were grown as 50-ml volumes to an OD_{540} of 0.70 in TYE containing gentamicin (10 mg/liter). Cells were harvested from 10 ml of cell culture by centrifugation at 8,000 × *g* for 5 min, washed, and resuspended in a mixture of 1 ml TYE (in the absence of gentamicin) plus 1 ml FCS. Cellulose discs (1 cm^2) were then inoculated with the test cell suspension, whereby each disc contained approximately the same number of viable cells (10^7 CFU/cm^2).

Viable counts. Microbe-laden test discs were placed and distributed evenly onto the surface of HTPs (eight per plate on eight plates) preincubated with FCS. Cut squares (25 by 25 mm) of each test wound dressing ($n = 16$ for each type) were then placed over the microbe-laden discs with each dressing type overlaying 16 discs across the surface of two HTPs. All assay plates (with discs and cut dressings) were incubated at 32°C for the duration of the experiments. Immediate samples (for time zero) and at various later sampling times over the course of 24 h (see Results for actual time points used) were taken by lifting wound dressing squares with the sequence of samples on a given plate determined according to a random clock generator, in order to remove test or control discs (using sterile forceps). Each sample disc was added to a 10-ml volume of sterile PBS containing 0.05% (wt/vol) $Na_2S_2O_3$ as an I_2 and Ag^+ neutralizer and vortex mixed for 1 min to release surviving cells into suspension. Neat suspensions were serially diluted in a 1:10 dilution series to 10^{-2} in PBS. Viable count samples of neat and diluted suspensions were then plated onto nutrient agar recovery medium containing gentamicin (10 mg/liter), using a spiral plater, to determine the numbers of survivors at different time points. Recovery plates were incubated at 37°C aerobically for 24 h, and the number of survivors was determined as CFU per sample (CFU/cm^2).

Bioluminescence (photon count). One FCS-preincubated HTP per test dressing and a control sample (prepared as described previously) were set up and inoculated, each with a single microbe-laden disc. The HTPs were then inverted and placed beneath an ICCD photon-counting camera mounted onto a blacked-out incubator set at 32°C. By inverting the HTP, the light emission from each test and control sample disc can be captured through the transparent hydrogel irrespective of the composition of the dressing applied to the HTP surface. Each test HTP was separated from every other by blackout material to minimize photon interference between samples. An initial photon count reading per disc was determined using a 60-s integration time with the camera set at 10% sensitivity. One cut square (25 by 25 mm) of each test wound dressing was then applied to a disc on an HTP, leaving one HTP and disc uncovered as the control. Photon count readings per disc were then recorded every 5 min for 24 h, using a 60-s integration time at each time point. The numbers of photons per sample were expressed as photons/cm^2 since the discs have a surface area of 1 cm^2.

Sample interference. To determine the effects of photon interference between samples, one FCS-preincubated HTP per test dressing and control sample were set up as described above. Only the control HTP was inoculated with a single microbe-laden disc. Photon count readings per dressing sample position (i.e., in the absence of microbes) were then recorded every 5 min for 24 h, enabling a base level of photon interference to be determined from the average of the three sample positions.

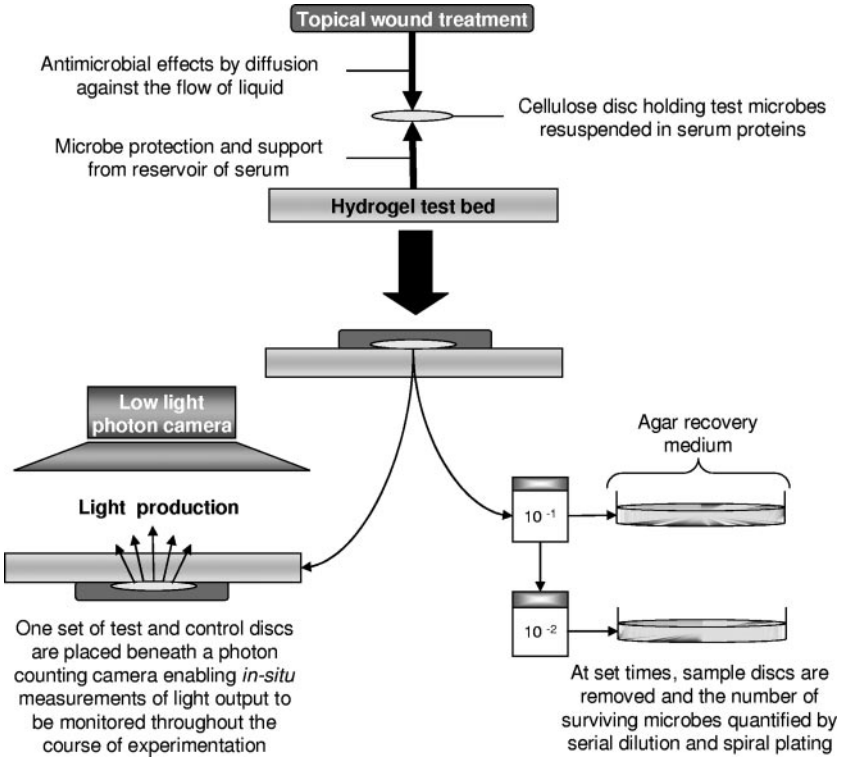

FIG. 1. Cross-section schematic diagram of the modified in vitro static diffusion method. Test organisms are held in a cellulose matrix, whereby the majority of cells are in close but indirect contact with the dressings under testing and are exposed to any antimicrobial effects for defined periods of time and under controlled physiochemical conditions. Kill rates can then be followed by enumeration of survivors or by monitoring light production from luminescent bacterial strains.

Log-fold dilution standards. A series of log-fold exponential-phase cell suspension dilutions were used to inoculate freshly prepared discs to give different cell densities per disc prior to placement onto HTPs. Photon count readings were immediately taken (using a low-light camera), and then the discs were removed to ascertain viable counts.

Alternative light capture. The antimicrobial efficacy of the three test wound dressings was again assessed, but with light output monitored by bench luminometry on extracted disc samples.

Data analysis. To determine if there was a significant correlation between bioluminescence and viable counts for each experiment, the two measurements were plotted against each other and a linear regression analysis was performed. Kill rates were determined for the three test wound dressings for both photon and viable counts by linear regression of the mean of three repeat experiments. An analysis of covariance was used to determine if there was a significant difference between these rates of kill. All repeat experimental data for both measurements were then used to establish if there was a significant linear correlation between bioluminescence (photon counts) and viable counts. An analysis of covariance was used to determine if this correlation was statistically different from that obtained for a series of log-fold dilution standards (prepared as described above). Graph construction, statistical analyses, and modeling were conducted with the use of GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA).

RESULTS

The planktonic growth curves obtained for the bioluminescent *P. aeruginosa* MCS5-lite showed differences in shape when following either viable counts or bioluminescence (Fig. 2a). During the first 2 h (lag phase), there was little increase in viable counts in contrast to a rapid increase in bioluminescence. Both parameters then appear to increase exponentially from 2 to 6 h (exponential growth phase). It is during this exponential phase of growth that there is good correlation between the two variables ($r^2 = 0.969$) (Fig. 2b). At the end of exponential growth, the light output of the cells plateaus around 2 h earlier than the viable counts (deceleration phase) and, interestingly, the 24-h samples (late stationary phase) show a lower light reading than this plateau (Fig. 2b).

Exponential-phase bacterial cell suspensions were then used to inoculate discs placed within the in vitro wound model and incubated to allow the growth kinetics to be observed in situ. The microbes appear to go through three distinct phases of growth during the first 12 h corresponding to the lag phase (0 to 2 h), exponential growth phase (2 to 7 h), and deceleration phase (7 to 12 h). The photon count was shown to closely follow viable counts over this period to near the maximum cell population that a disc can support (Fig. 3a). As with growth in planktonic culture (Fig. 2a), the 24 h samples show a lower light reading than the photon plateau reached during the first 12 h of growth. When all data for both photon and viable counts (excluding 24-h data) were plotted against each other for cells grown in the disc, there was a significant linear correlation (Fig. 3b) ($r^2 = 0.982$). The slope of the relationship differs significantly ($P < 0.0001$) from the correlation obtained for a series of log-fold dilution standards of exponential-phase cells, prepared to give different cell densities per disc (Fig. 3b).

A series of experiments was designed to assess the relationship between bioluminescence and viable counts for cells within a killing/inhibitory environment. These in vitro wound model experiments were performed based on the data from the

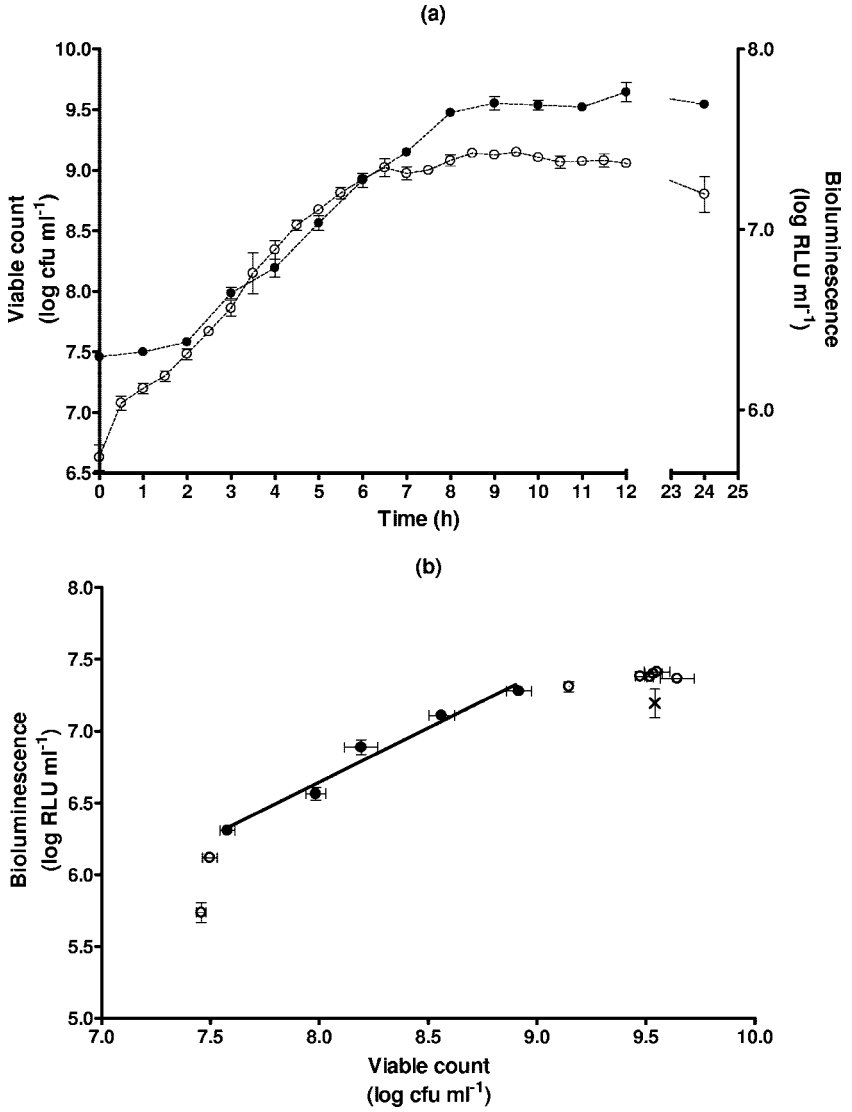

FIG. 2. (a) Planktonic growth curves of bioluminescent *P. aeruginosa* as followed by viable counting (●) and bioluminescence (○) over a 24-h period. (b) Relationship between viable counts and bioluminescence during growth (○). The correlation between the two variables during the exponential growth phase (2 to 6 h) is highlighted (●), as is the 24-h sample (X). Symbols represent the mean of four experiments and error bars the standard deviation. RLU, relative light units.

initial growth experiments that showed there was a good correlation between bioluminescence and viable counts for exponential-phase cells growing in planktonic culture and also when these cells were integrated into a growing environment within the in vitro wound model. Therefore, cell cultures for inoculating cellulose discs were grown up to a standardized OD_{540} of 0.70 in planktonic culture (around 5 h of growth and still within the exponential growth phase), a point at which there were sufficient numbers of cells to perform accurate and meaningful kill curves.

Figure 4a shows the kill curves obtained from exposure of *P. aeruginosa* MCS5-lite to three types of antimicrobial wound dressing and followed by viable counting of survivors in discs. It can be seen that dressing 2 elicits the highest rate of kill, followed by dressing 1 and dressing 3 (Table 1), with all kill rates being significantly different both from each other and from the control ($P < 0.0001$). Figure 4b shows the kill curves obtained when following photon counts in discs, and the order of efficacy for the three test dressings was the same as that determined from viable counts (Table 1). Some differences exist between the two measurements: for example, within control discs when following photon counts the light output started to decrease after around 6 h of experimentation (Fig. 4b), in contrast to viable counts, which remained relatively stable (Fig. 4a). By 24 h, there was significantly less light produced per cell than would be expected for the cell numbers determined by viable counting if there was a constant correlation between the two measurements over this time period. Once a dressing elicits a sufficient kill rate, the photon count appears to reach a base-level plateau of around 10^2 photons/cm^2 due to a combination of background photon counts and sample interference.

When all of the in vitro wound model data for both photon

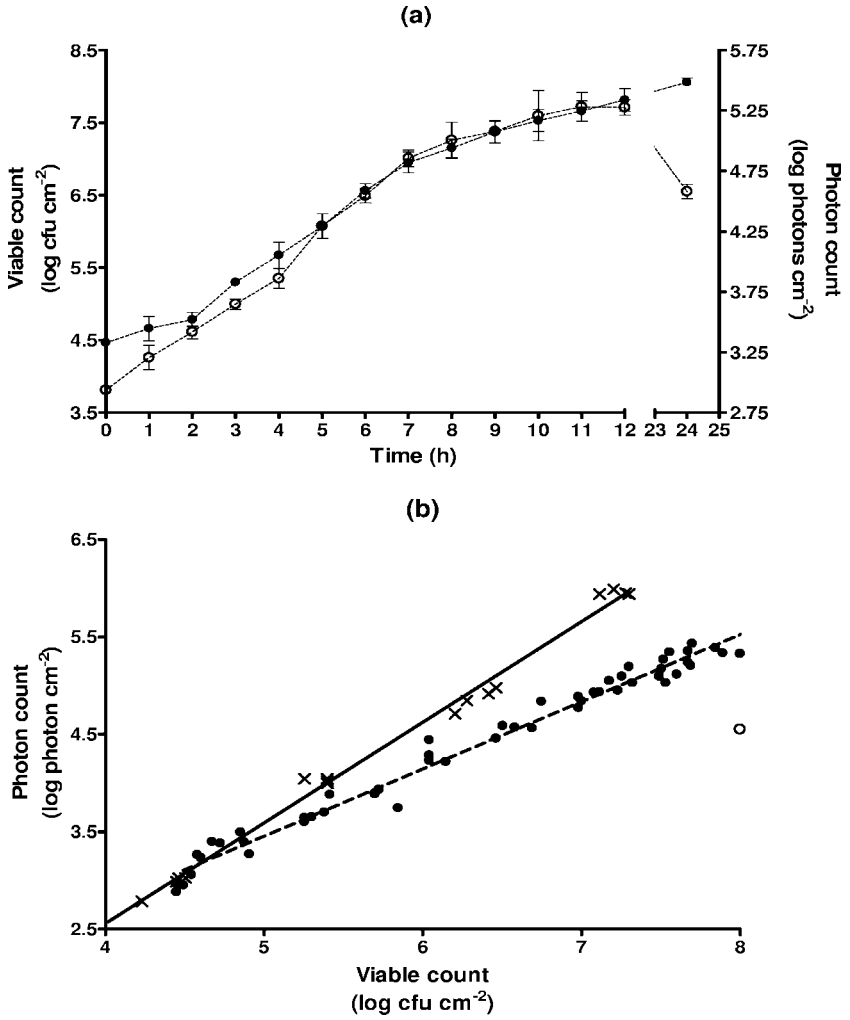

FIG. 3. (a) Growth curves of *P. aeruginosa* MCS5-lite grown in discs within the in vitro wound model, as followed by bioluminescence (photon counts) (○) and viable counting (●). Symbols represent the mean of four experiments and error bars the standard deviation. (b) Linear regression analysis of the correlation between photon and viable counts for *P. aeruginosa* MCS5-lite during growth in discs (all plots from data in panel a) (● and dotted line regression), compared to a log-fold dilution standard prepared to give different cell densities per disc (X and solid line regression).

and viable counts relating to the antimicrobial efficacy of the three wound dressings are plotted against each other (i.e., within a killing environment), there is a significant linear correlation ($r^2 = 0.873$) (Fig. 5a). Furthermore, the relationship between photon and viable counts is stronger if the rates of kill for each agent data set determined from viable counts are plotted against the rates of kill for each agent data set determined from photon counts ($r^2 = 0.982$) (data not shown). This correlation is significantly different ($P < 0.0001$) from that obtained for a series of log-fold dilution standards of exponential-phase cells, prepared to give different cell densities per disc in the absence of an antimicrobial agent.

The above assessment of the antimicrobial efficacy of three wound dressings was repeated, but this time bioluminescence was measured using a single-tube luminometer in which test cell suspensions were first extracted from the sample discs. Again there is a significant correlation between bioluminescence and viable counts ($r^2 = 0.957$) (Fig. 5b), and this is significantly different ($P < 0.0001$) from that obtained for a series of log-fold dilution standards in the absence of an antimicrobial agent.

DISCUSSION

There is a good correlation between viable counts and bioluminescence during the exponential growth phase (2 to 6 h) in planktonic culture but not in the lag (0 to 2 h), deceleration (6 to 9 h), stationary (9 to 12 h), or late stationary (24 h) phase (Fig. 2b). This growth-phase-dependent condition has been shown in previous in vitro studies using bioluminescent bacterial reporters (21, 25). The initial increase in bioluminescence compared to viable counts, seen within the planktonic growth curve kinetics of *P. aeruginosa* during the lag phase (0 to 2 h; Fig. 2a), can be explained by considering the different cell properties that the two methods measure. Bioluminescence increases in accordance with the adenylate energy charge and

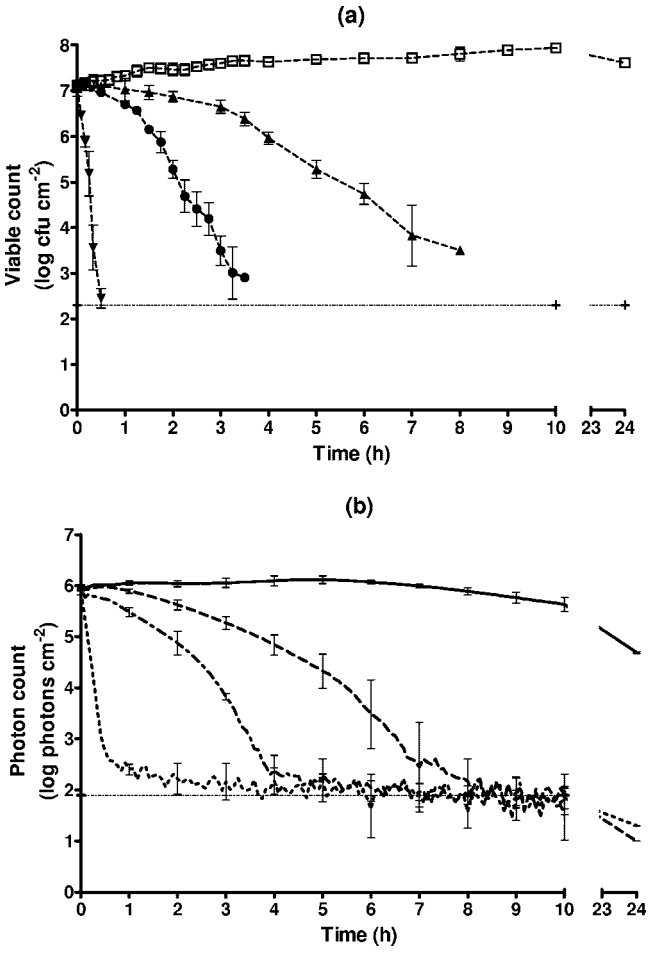

FIG. 4. Kill curves obtained for bioluminescent *P. aeruginosa* strain MCS5-lite treated with three types of antimicrobial wound dressings over a 24-h period followed by viable counting (a) and bioluminescence (photon counts) (b). Shown are results for the untreated control (□ and solid line), dressing 1 (● and hatched line), dressing 2 (▼ and dotted line), and dressing 3 (▲ and dashed line) and the minimum detection level (+). Symbols (viable count) and lines (bioluminescence) represent the mean of three experiments and error bars the standard deviation (shown only hourly for bioluminescence).

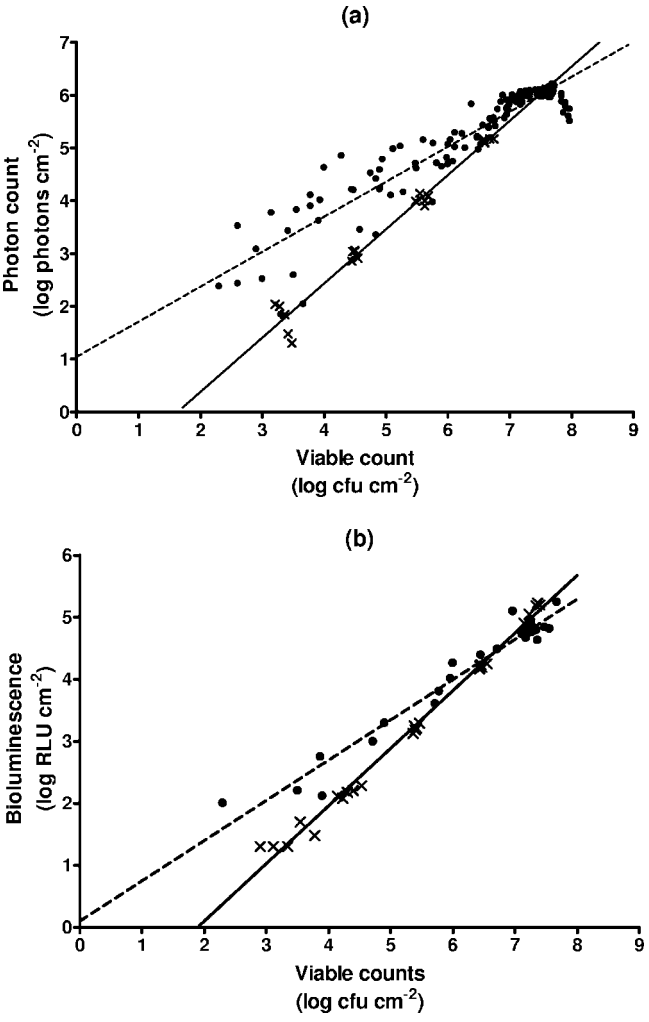

FIG. 5. (a) Linear regression analysis of the correlation between viable counts and bioluminescence (photon counts) determined in situ within a killing environment (● and dotted line regression [data from all experiments used to create the plot in Fig. 4]) compared to that determined for a log-fold dilution standard series prepared to give different cell densities per disc in the absence of an antimicrobial agent (X and solid line regression). (b) Linear regression analysis of the correlation between viable counts and bioluminescence determined after extracting the cells from the wound model (● and dotted line regression) compared to that determined for a log-fold dilution standard series (as described above) (X and solid line regression). RLU, relative light units.

the availability of reducing power within the cell (i.e., its metabolic state) (9). However, it is not until sufficient intracellular components have been synthesized (e.g., ribosomes and cell polymer building blocks and precursors) that the cells will begin to divide (26): hence the lag in the increase in viable cell numbers but not in metabolism or bioluminescence. This initial increase in bioluminescence compared to viable counts was also observed for cells grown in cellulose discs within the in vitro wound model for the same reasons (Fig. 3a), albeit to a lesser extent.

TABLE 1. Kill rates and regression r^2 values for three antimicrobial wound dressings tested against a bioluminescent *P. aeruginosa* strain over a 24-h period[a]

Wound dressing	Viable counts		Photon counts	
	Kill rate (log reduction/h)	r^2	Kill rate (log reduction/h)	r^2
1	1.328	0.911	0.835	0.916
2	9.787	0.924	6.501	0.899
3	0.472	0.898	0.446	0.856

[a] Rates were determined by both viable counts (CFU/cm^2) and bioluminescence (photons/cm^2).

Light output from the bacterial cells grown in planktonic culture plateaus around 2 h earlier than the viable counts, and we hypothesize that this is due to ADLA as the bacterial cell population starts to decelerate and enter into stationary phase (Fig. 2a). Although the number of cells is still increasing, it is likely that the metabolic rate of the cells in general begins to slow down as nutrients become depleted. In fact, it has been theorized that ADLA is caused by a programmed process

causing depletion of the available pool of reducing power NAD(P)H, as a necessity for differentiation into stationary-phase cells (19). At 24 h, both for cells grown in planktonic culture and for those cells grown in discs within the in vitro wound model, there was considerably less light produced than would be expected if there was a constant correlation between bioluminescence and viable counts over this time period. This was likely a consequence of the nutrient-starved conditions of a late-stationary-phase culture in which there will be a depleted pool of reducing power available for bioluminescence, even though many cells retain viability and can be recovered on plates. To further investigate this phenomenon, microbe-laden discs grown up for 24 h within the in vitro wound model were transferred to new HTPs and refreshed with additional nutrients. It was found that light production increased rapidly, reaching a peak level equal to that of discs freshly inoculated with exponential-phase bacterial cells (data not shown). This highlights the effect of nutrient limitation on the level of bioluminescence within bacterial cells.

The correlation between bioluminescence (photon counts) and viable counts for the bacterial cell population grown in cellulose discs is significantly linear throughout the first 12 h of growth, and hence this correlation appears to be largely irrespective of the growth phase during this period (Fig. 3b). It is unlikely, however, that the true stationary growth phase has been reached in this time course; thus, a linear correlation would be expected. When this linear correlation is compared to that obtained for a series of log-fold dilution standards, prepared to give different cell densities per disc, it is evident that significantly less light is produced per cell during growth in the disc within the in vitro wound model (Fig. 3b). As expected, the initial proportion of photons to viable counts is the same (i.e., when the exponential-phase cells are first resuspended into discs); however, as growth progresses, the discrepancy between the two correlations increases. Initially this phenomenon was thought to be due to the cells grown in the discs having a lower metabolic rate; however, it was actually found that these cells had a faster growth rate than the planktonic cell population used in this comparative experiment. An alternative hypothesis is that the cells within the cellulose discs are likely to go through sequential development towards a simple biofilm phenotype. Therefore, although the number of cells is rising, an increasing proportion of these are likely to be immobilized within the core of microcolonies making up the biofilm architecture. It is possible that the light output from bacterial cells within these microcolonies will be attenuated by surrounding cells due to absorption (even though these cells are themselves emitting light). Hence, the greater the density of cells grown in the disc, the more "light-insulated" cells there will be within microcolonies and the greater the discrepancy will be between viable counts and photons, as is observed.

As has already been discussed, due to the results of these initial experiments an exponential-phase culture of *P. aeruginosa* MCS5-lite was used for integration into the wound model to assess the relationship between bioluminescence (photon counts) and viable counts in a killing environment. All three antimicrobial wound dressings tested elicited kill rates that were significantly different from those of the control and each other ($P < 0.0001$), whether following viable or photon counts. Interestingly, there was a slight but significant ($P < 0.0001$) decrease in photon counts after approximately 6 h for the control samples, compared to increasing numbers as monitored by viable counting. This was most likely caused by the effects of nutrient depletion as discussed previously. As a control would always be included with any analysis within this model, this would not affect the ability to determine whether any given dressing was having a significant antimicrobial effect.

The amount of photon interference from a control microbe-inoculated disc was experimentally determined to be, on average, 8.18×10^1 photons cm^{-2} per test sample position (shown as the minimum detection level in Fig. 4b). The level of photon interference within the assay was in the same order as this value, although it appears to have a greater effect during the first few hours. This can be explained by the added interference caused by the light emitted by adjacent test samples; hence, dressing 2 reaches a plateau slightly above the predicted interference base level due to the added effects of the dressing 1 and dressing 3 samples. The kill rates are, however, still easily discernible.

Both photon and viable counts produced characteristic kill curves relating to the inactivation kinetics (pharmacodynamics) of the agents released from the wound dressings. However, when monitoring photon counts, the in situ measurements allowed quantitative data to be obtained more rapidly. This is a consequence of the sample readings being both nondisruptive and being taken much more frequently (i.e., every 5 min) from the same undisturbed sample disc and monitoring a variable (light production) that gives an immediate numerical output.

The order of efficacies for the three test wound dressings was the same whether following photons or viable counts. This shows the close relationship between the two measurements in a killing environment and is reinforced by the significant linear correlation obtained when both measurements were plotted against each other (Fig. 5a). The correlation has been shown to be reproducible and holds for at least 4-log-fold drops in viable counts, well above the sensitivity deemed sufficient to monitor bactericidal agents (14). When this correlation between photons and viable counts in a killing environment is compared to a series of log-fold dilution standards, prepared to give different cell densities per disc in the absence of an antimicrobial agent, there was a significant difference ($P < 0.0001$) (Fig. 5a). This suggests that more light is produced per cell in a killing environment within the discs (i.e., in the presence of the antimicrobial agent). This discrepancy is partly accounted for by the effect of photon interference between low-light test and high-light control samples, as all samples were comonitored by the same low-light photon camera. To determine whether this observation was the result of taking bioluminescence readings in situ within the wound model, the experiment was repeated but with bioluminescence readings taken using a single-tube luminometer. This involved removing sample discs and extracting the cell suspension before measuring light output, thereby removing any interference between sample readings and any other artifacts that may be caused by measuring light output in situ. A significant linear correlation was found between bioluminescence and viable counts (Fig. 5b), and again this differed significantly from the correlation obtained for a series of log-fold dilution standards. This provides further proof that more light is produced per cell in a killing environment within the discs. One possible explanation is that the replicative machinery of a cell can be irreversibly damaged by an antimicrobial agent so that it is unable to recover viability when plated onto

appropriate medium; however, it is still able to produce light. This phenomenon has been previously hypothesized (27). Furthermore, it has been shown that under certain conditions of stress (e.g., UV radiation) both naturally bioluminescent wild-type species (7) and genetically constructed strains can produce more light (6). This is thought to be due to the bacterial cells using the bioluminescent reaction pathways both to eliminate harmful reactive oxygen species (8) and to aid DNA repair (29), and it could be a contributing factor to the observation in this study that more light is produced per cell in a killing environment.

Plate growth recovery techniques, such as zone of inhibition (24) or time-kill curves (15), have long been used as the gold standard for determining the antimicrobial efficacy of wound dressings in vitro. Here it has been shown that it is possible to integrate a bioluminescent *P. aeruginosa* into a modified version of an existing in vitro microbiological system, as a method for assessing antimicrobial wound dressing efficacy, and that there is a good correlation between bioluminescence readings and viable counting under defined conditions. Although this relationship alters, depending on the physicochemical environment to which the cells are exposed, as long as defined conditions are employed (i.e., a known starting population density of exponential-phase cells grown up in planktonic culture before integration into the in vitro wound model), then a significant linear correlation is observed. This enables the state of the system to be continually monitored without disturbance, allowing more immediate and accurate calculations of kill rates without the need for viable counting. Within the field of wound care, this could allow antimicrobial wound dressings to be tested in a high-throughput, low-cost manner (after the initial expenditure on equipment) and greatly enhance the efficiency by which either conventional or prototype wound dressings could be assessed, possibly bringing prototype dressings to the market in a shorter time span.

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